AD_____

Award Number: W81XWH-11-1-0562

TITLE: A Novel Approach for Preventing HIV Infection and Reducing Risk to U.S. Military Personnel

PRINCIPAL INVESTIGATOR: Warner C. Greene, M.D., Ph.D.

CONTRACTING ORGANIZATION: The J. David Gladstone Institutes
San Francisco, CA 94158-2261

REPORT DATE: September 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2013	Annual	10 August 2012-09 August 2013

4. TITLE AND SUBTITLE

A Novel Approach for Preventing HIV Infection and Reducing Risk to U.S. Military Personnel

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0562

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Warner C. Greene, M.D., Ph.D.

E-Mail: wgreene@gladstone.ucsf.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

The J. David Gladstone Institutes
San Francisco, CA 94158-2261

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The most common mode of HIV transmission is through sexual contact, and as such semen is the vector that is fueling the worldwide spread of the virus. We and our colleagues have identified and characterized amyloid fibrils from human semen that substantially promote HIV infection. In our funded proposal, we describe experiments to study the components of semen that enhance HIV infection, to decipher their mechanisms of action both *in vitro* and *in vivo*, and to identify methods of blocking their activity. We have made significant progress in our first year of the funded proposal. In addition to identifying a novel set of viral enhancing factors from semen, we have identified new mechanisms by which these amyloids can promote HIV transmission. We have shown an association between the levels of semen amyloids with endogenous viral load in samples form HIV-infected men, and demonstrated the activity of the amyloids *in vivo* in a humanized mouse model of HIV transmission. Finally, we have initiated a small-molecule screen for inhibitors of these amyloids, and have identified promising hits that we are currently further testing in secondary analysis. Inhibiting the activity of these semen factors can lead to the development of a new generation of microbicides targeting HIV together with naturally-occurring viral enhancement factors.

15. SUBJECT TERMS
HIV, AIDS, Transmission, Semen, SEVI (Semen-derived Enhancer of Viral Infection), SEM (Semenogelin), Amyloid fibrils, Animal model, Infection, Inhibitors, Inflammation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	24	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Our proposal “A Novel Approach for Preventing HIV Infection and Reducing Risk to US Military Personnel” focuses on understanding the molecular mechanisms by which semen enhances HIV infection, with the ultimate goal of developing specific inhibitors of this activity. Data emerging over the past five years have demonstrated that semen can markedly enhance HIV-1 infection *in vitro* (1-10). The first factor identified from semen that was sufficient to enhance HIV infection was termed Semen-derived Enhancer of Viral Infection [SEVI], cationic amyloid fibrils that promote the attachment of HIV-1 virions to their cellular targets (2, 4). As described in Year 1 of our annual report, we identified a second amyloid fibril from human semen, made up of fragments from the N-terminus of semenogelin [SEM], the major constituent of the semen coagulum (8). Significantly, semen samples naturally deficient in SEMs lack the ability to enhance HIV infection, suggesting that these proteins are key for the viral enhancing activity of semen (8). During our second year of funding, we focused our research efforts on understanding the regulation of semen amyloid activity over time, in studying the interplay between semen amyloids and other factors naturally present in semen, and in examining the effects of semen and semen amyloids on the female genital tract. We also completed a primary screen for inhibitors of semen amyloids, which we plan to advance through secondary and tertiary analyses (pending a third year of TATRC research funding), with the ultimate goal of developing compounds that when incorporated into microbicides will decrease HIV transmission rates by blocking semen-mediated enhancement of HIV infection.

BODY

We describe in this section the research accomplishments for Year 2 of the proposal.

A. Detailed characterization of the viral enhancing activity of semen

We previously reported that the ability of semen to enhance HIV infection decreases with increasing time of liquefaction (6). However, the mechanism(s) underlying this progressive decrease in viral enhancing activity was unclear. Semen naturally undergoes a process termed liquefaction, during which the viscous coagulum becomes more fluid. In these studies, we set out to characterize the mechanisms underlying the loss of enhancing activity during prolonged periods of liquefaction.

"Fresh" semen exhibits potent enhancing activity that decreases with increasing times of liquefaction

We first set out to examine the effect of "fresh" ejaculates (that have not undergone any freeze/thaw cycles or other processing steps) on HIV-1 infection, as this would most closely mimic semen's effect during natural transmission. Ejaculate was collected and liquefied for 10 minutes, at which point it became pipetable. An aliquot of the sample was then mixed with HIV-1 and added to TZM-bl reporter cells. Samples were also taken at later timepoints following initiation of liquefaction and analyzed in a similar fashion. As shown in Fig. 1, a reduction in enhancement activity was observed with increasing liquefaction times, but enhancing activity remained even after 150 minutes. These results suggest that fresh ejaculates potently enhance HIV infection, but that this activity is progressively lost during prolonged periods of liquefaction.

Prolonged liquefaction leads to a degradation of SEM fragments and reduces the ability of semen to enhance HIV infection

We next attempted to understand how prolonged liquefaction diminishes the viral enhancing activity of semen. Experiments with fresh ejaculates like those used in Fig. 1 are logistically challenging because it is often difficult to time sample collection with the initiation of the assay, plus the volume from a single ejaculate is not sufficient for multiple assays. To overcome these issues, we generated a stock solution of seminal fluid for the subsequent assays. Use of seminal fluid instead of semen allowed us to study the effect of enhancing factors in the absence of spermatozoa and debris present in semen. To generate the seminal fluid stock, twenty semen samples from different donors were liquefied for 2 hr at room temperature prior to being frozen, and then simultaneously thawed, pooled, and centrifuged to remove spermatozoa. The supernatant was then aliquoted and frozen and served as our seminal fluid stock. To determine whether extending the liquefaction period affects its ability to enhance HIV infection, the stock was thawed and incubated for an additional 0.5, 2, 4, 8, or 24 hr at 37°C before being frozen. When the entire time-course was completed, all samples were thawed and tested simultaneously for the ability to enhance HIV infection of TZM-bl cells.

As shown in Fig. 2A, seminal fluid stock incubated at 37°C progressively lost the ability to enhance HIV infection. Enhancing activity was observed with seminal fluid samples incubated for 0, 0.5, and 2 hr, but not those incubated for longer periods of time. We performed Western blot analysis to determine whether the loss in viral enhancing activity correlated with the degradation of SEMs, the predominant component of the semen coagulum that is progressively cleaved during liquefaction. Semen samples from Fig. 2A were immunoblotted for SEM using commercially available antibodies recognizing both SEM1 and SEM2 isoforms, as well as antibodies generated against SEM1 (α-S1) or SEM2 (α-S2) fibrils (8). Multiple SEM fragments were readily detected in the seminal fluid stock solution, but their levels gradually decreased over the time-course, with large fragments becoming degraded before smaller fragments (Fig. 2B). Mass spectrometry confirmed the degradation of amyloidogenic SEM fragments upon prolonged liquefaction (data not shown). The degradation of SEM fragments closely parallels the loss of viral enhancing activity in these samples (Fig. 2A). The loss of SEM fragments over time is specific, as the levels of the semen protein albumin is constant over this time-course (Fig. 2B, *bottom*).

Inhibition of serine proteases in semen rescues its ability to enhance HIV infection following prolonged liquefaction

We next tested whether protease inhibitors prevent the disappearance of the viral enhancing activity of semen during prolonged liquefaction. We explored the effects of AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), a serine protease inhibitor that blocks the activity of Prostate Specific Antigen [PSA], the dominant protease in semen (11). AEBSF rescued the degradation of SEMs, and this corresponded with a rescue in HIV enhancing activity (Fig. 3). Interestingly, enhancing activity after AEBSF treatment was better than that of the starting material (i.e., seminal fluid stock that was not incubated at 37°C), possibly due to formation of new amyloids by the remaining SEM fragments over time. Addition of AEBSF in the absence of seminal fluid did not increase HIV infection (data not shown), indicating that the protease inhibitor was acting on factors in seminal fluid and not directly on HIV virions. These results suggest that preventing SEM degradation rescues the ability of semen to enhance HIV infection despite prolonged periods of incubation.

To summarize the results from this section, we investigated here the impact of liquefaction on the ability of semen to enhance HIV infection. We find that: 1) fresh semen (that was never before frozen) enhances HIV infection, and increasing the liquefaction period decreases the viral enhancing activity of the ejaculate, 2) the loss of enhancement activity during prolonged liquefaction parallels the degradation of amyloidogenic SEM fragments, 3) inhibiting semen proteolysis during prolonged liquefaction rescues viral enhancement activity. Our prior studies demonstrated that semen samples from different donors enhance HIV infection to different extents (6, 8). More recently, it was shown that longitudinal semen samples isolated from the same donor exhibit marked variability in viral enhancing activity (10) (our unpublished data). Our current findings provide another reason for sample variability relating to how long the

semen sample was allowed to liquefy. As Simian Immunodeficiency Virus (SIV) infects cells of the vaginal mucosa within 60 min of vaginal exposure (12), a period of time wherein fresh ejaculates harbor potent viral enhancing activity, we believe that semen has ample opportunity to establish the initial beachhead of infection following HIV exposure and promote transmission within the genital mucosa. Together, these data underscore the heterogeneity and fluctuation of the viral enhancing activity of semen. As such, it will be important to ensure that inhibitors targeting this activity (see section below) are effective against semen samples with differing levels of viral enhancement, before such compounds are considered as a component of future combination microbicides that attack both the virus and enhancing factors present in semen.

B. Exploring the effects of semen and semen amyloids on genital epithelial cells and *in vivo* to model semen-mediated HIV transmission

In addition to directly promoting HIV infection by facilitating viral attachment to target cells, semen amyloids may also indirectly promote HIV transmission by inducing inflammation. Amyloid fibrils generally induce TLR signaling, inflammasome activation, and NF-κB activation(13-16). Semen itself has also long been documented to be inflammatory, and can stimulate epithelial cells to produce inflammatory cytokines including IL-6, IL-1β, IL-8, and MCP-1(17-19). An inflammatory environment can upregulate HIV gene expression driven by NF-κB and the secretion of chemokines that recruit HIV-permissive cells(20-23). Inflammatory cytokines including TNF-α, IL-6, IFN-γ, and IL-4 can also increase the permeability of the mucosal epithelium, thereby promoting viral translocation from the apical to the basolateral surface(24-27).

Although both amyloids and semen have long been documented to be inflammatory, and inflammation has been linked to increased HIV transmission, the direct link between semen amyloids and increased HIV transmission has not been established. To better understand the inflammatory cytokines induced by semen and semen amyloids in the female reproductive tract (FRT), we assessed the response of primary endometrial epithelial cells and stromal fibroblasts to seminal fluid and SEM1 amyloids by microarray. We initially focused on the endometrium because its single cell layer of columnar epithelium may be an especially vulnerable site of viral attack, in contrast to cells of the lower FRT (vagina and ectocervix), which are distinguished by a thick layer of stratified squamous epithelium. Significantly, although microarray analysis has previously been carried out on semen-treated ectocervical epithelial cell lines, with selected induced genes confirmed in primary cells(19), to our knowledge our analysis is the first to examine the global response of *primary* human FRT cells to semen components.

Exposure of both epithelial and stromal cells to 1% seminal fluid for 6 h (conditions that did not elicit cytotoxicity (data not shown)) induced the expression of pro-inflammatory gene products like IL-6, confirming prior studies of the pro-inflammatory effects of semen(17-19). Although IL-6 mRNA was not induced by purified SEM amyloids, we found efficient induction of C1q, a subcomponent of complement that triggers IL-6 production(28). We therefore

measured production of IL-6 directly by ELISA after 24 h, and found that SEM amyloids induced secretion of IL-6 in primary endometrial stromal fibroblasts. These data suggest that both SP and SEM amyloids can induce production of pro-inflammatory cytokines including IL-6. Year 3 studies will investigate whether the levels of IL-6 induced are sufficient to enhance HIV replication and gene transcription in permissive cells.

As discussed in the Year 1 annual report, we also conducted preliminary *in vivo* experiments on the effects of semen amyloid on vaginal transmission of HIV-1 in humanized mice. In collaboration with Drs. Shomyseh Sanjabi, we obtained a colony of MISTRG humanized mice that were engrafted with human fetal liver. Mice that were infected with HIV in the presence of SEVI had a higher percentage of p24+ CD4+ T cells. In addition, the addition of SEVI during infection increased the mean fluorescence of intracellular p24 in CD4+ T cells in HIV-infected mice. These preliminary results, detailed in the Year 1 report, suggest that HIV-1 establishes infection in MISTRG mice, and that SEVI enhances this process. Pending third year of research funding, we will examine the effects of semen on HIV transmission in this system, and test promising inhibitors (described in the last section of this segment) in this model.

C. Fibronectin augments amyloid-mediated enhancement of HIV infection

Seminal amyloids do not act alone, but rather in concert with other factors in semen. To determine whether the viral enhancing activity of semen amyloids can be modulated by naturally-occurring factors in semen, we set out to identify interacting proteins of the SEM1 amyloids by carrying out a pull-down experiment. Biotinylated SEM amyloids, whose amyloidogenic properties and HIV-enhancing activity were confirmed by Thioflavin T binding, electron microscopy, and infectivity assays (data not shown), were conjugated to streptavidin-conjugated MyOne^{TM} beads (Invitrogen) and then incubated with a 20% solution of seminal fluid for 2 hr at 4°C. Following extensive washing, samples were digested by trypsin and analyzed by mass spectrometry (electrospray ionization-liquid chromatography-tandem mass spectrometry, or ESI LC-MS/MS). When the raw MS data were searched against the SwissProt human database using SEQUEST, the top high-confidence interactor of the amyloids was identified as fibronectin (FN), a major component of the semen coagulum. The levels of seminal FN bound to control samples containing beads only or the corresponding monomeric SEM peptide was significantly less than that bound to the fibrils (Fig. 4A). Dot and Western blot analyses confirmed that purified FN physically interacted with SEM amyloids (Fig. 4B). The ability of FN to bind semen amyloids is consistent with the previous reported ability of this protein to bind amyloid fibrils including bacterial curli (29).

We then assessed whether the binding of SEM amyloids to FN affects the ability of the fibrils to enhance HIV infection. Infection of TZM-bl cells with CCR5-tropic 81A HIV-1 was carried out as previously described (8). In brief, semen factors were pre-incubated for 5 min with HIV-1 (100 ng/ml $p24^{Gag}$) at the indicated concentrations, and then diluted 15-fold and added to TZM-bl cells. Infection levels were assessed three days later by monitoring β-galactosidase

activity. FN on its own inhibited HIV infection (Fig. 5A), consistent with prior reports of FN inhibiting HIV infection by inhibiting the interaction between gp120 and the CD4 receptor (30). The inhibition of HIV infection by FN was not the result of cytotoxicity, as viability of FN-treated cells, as assessed by ATP levels, was not lower than that of mock-treated cells (data not shown). Strikingly, addition of FN to SEM amyloids led to viral enhancement activity that was better the enhancement induced by SEM amyloids alone (Fig. 5B). A synergistic effect between the two factors was demonstrated by a dose-response curve, wherein the theoretical sum of infection levels in the presence of either factor alone was markedly less than that observed experimentally (Fig. 5C). Synergistic enhancement of HIV infection was also observed between FN and SEVI amyloids (Fig. 5D), suggesting that FN may generally boost the viral enhancing activity of cationic semen amyloids. Importantly, however, FN did not synergize with human albumin to enhance HIV infection (Fig. 5E), demonstrating that its effects were specific to the amyloids. Furthermore, the SEM and SEVI amyloids did not synergize with each other (Fig. 5F), demonstrating that synergy only occurs between FN and semen amyloids. The synergistic enhancing effects between FN and the amyloids was not due to increased proliferation of the cells, as ATP levels were not affected by the presence of these factors (data not shown). FN similarly boosted the ability of semen amyloids to enhance HIV-1 infection of the T/B hybridoma CEM.M7 reporter cell line and primary CD14-CD4+ PBLs (activated with PHA and IL-2 48 hr before infection with CCR5- or CXCR4-tropic nef-IRES-eGFP HIV-1), demonstrating that the synergy also occurred in non-adherent and primary cells, and that the effect was observed with both R5- and X4-tropic viruses (Fig. 5G,H, Fig. 6).

We then determined whether FN altered the structure of the amyloids. By electron microscopy (EM), no marked alterations in SEM amyloid structure were observed in the presence of FN (Fig. 7A). To determine whether FN increased the ability of semen amyloids to bind HIV-1, we carried out a viral pull-down assay (5). SEM amyloids (100 μg/ml) in the absence or presence of FN or albumin (100 μg/ml) were incubated with HIV-1 81A (100 ng/ml $p24^{Gag}$), pelleted by low-speed centrifugation (13,000 rpm, 5 min), and assessed by $p24^{Gag}$ ELISA for the fraction of pelleted virions. As shown in Fig. 7B, FN did not significantly increase the ability of SEM amyloids to bind and pellet HIV-1, suggesting that FN did not promote amyloid-mediated enhancement of HIV infection by increasing the number of bound virions. These data argue against the notion that FN increases HIV infection by altering the fibrils in a way that facilitates their binding to HIV-1 virions. FN also did not increase the binding of SEM fibrils (visualized by Proteostat) to TZM-bl cells (visualized by DAPI) (Fig. 7C). Together, these data suggest that FN increases the ability of semen amyloids to enhance HIV infection through mechanisms other than promoting amyloid attachment to virions and target cells.

Although HIV-enhancing semen amyloids have been well-characterized for their ability to enhance HIV-1 infection (2, 4) (7-10), their ability to bind and be modulated by other proteins in semen has not been studied. Here, we identify FN as a binding partner of HIV-enhancing SEM amyloids. Binding of FN to both SEM

and SEVI amyloids synergistically increases their ability to enhance HIV-1 infection, and may explain the marked ability of semen (which contains all three factors) to enhance HIV-1 infection *in vitro* (2, 6). The mechanism by which FN augments the activity of the amyloids is unclear, but does not appear to be mediated by increased viral attachment. The studies described in this section report an unprecedented ability of FN to further augment the HIV-enhancing activity of semen amyloids, and suggest that targeting the interface between semen amyloids and FN could be a novel microbicide strategy.

D. Completion of primary screen for deaggregators of semen amyloid

We set up a small-molecule screen at the Small Molecule Discovery Center (SMDC) at UCSF. As mentioned in prior progress reports, we previously conducted a pilot screen of 2,688 bio-active compounds to identify molecules that can disassemble SEM1 amyloids. Although we identified some compounds with activity, subsequent tests showed that the activity was not potent and reproducible. Instead of focusing on the pilot screen, we moved forward with the full screen of testing 137,872 compounds for their ability to disassemble SEM amyloids. A final compound concentration of 20 μM in 0.1% DMSO was added to pre-formed amyloids. After a 2 hr incubation, 5 μM Thioflavin T (ThT) was added. Because ThT emits strong fluorescence at 482 nm only when bound to amyloid fibrils, we screened for compounds that decreased the ThT signal in the presence of the amyloids. Results from the initial screen were shown in the third Quarterly Report for Year 2. We used ThT-treated amyloids in the presence of DMSO as negative controls, and ThT in the absence of amyloids as positive controls. Assay wells with calculated inhibition exceeding the Mean + 3xSD (68%) formed the initial hit list (775 compounds, 0.57% hit-rate). These were prioritized according to their percentage inhibition. The top 320 (>84% inhibition) were cherry-picked for counter-screening for gross fluorescence quenching against 1μM fluorescein, in an effort to flag potential false-positives. The remaining 305 compounds were then tested in dose-response against the primary screening assay (ThT displacement), and a four-parameter logistical fit was used to determine EC_{50} and Hill slope (cooperativity). This analysis identified 67 compounds with inhibitory Hill coefficient slopes between 1 and 2.5, suggestive of a cooperative interaction between ligand and target, a desired property for inhibitors. However, as aggregation kinetics may deviate from that of standard ligand/target cooperativity, we will not rule out candidates whose Hill coefficients were <1 or >2.5; however, these compounds will be tested at a lower priority than compounds with coefficients between 1 and 2.5. Pursuit of hit compounds from the primary screen will be carried out pending Year 3 of proposal funding. These investigations would include testing hit compounds in the *in vivo* murine model of vaginal infection with HIV-1 described earlier.

KEY RESEARCH ACCOMPLISHMENTS FOR YEARS 1&2

- Identification of new factor from semen that enhances HIV infection. Identification and characterization of this factor was published in a manuscript entitled “Peptides released by physiological cleavage of semen coagulum proteins form amyloids that enhance HIV infection” (Cell Host and Microbe. 2011 10(6)541)

- Semen samples from HIV-infected men enhance HIV infection, and semen viral load in these samples correlates with the levels of semen amyloid

- Semen and semen fibrils induce an inflammatory response, including production of IL-6, in genital tract epithelial cells

- Semen fibrils enhance vaginal transmission in a humanized mouse model of HIV infection

- The abundance of SEM amyloids and the ability of semen to enhance HIV-1 infection decrease progressively over the course of liquefaction

- Semen amyloids are associated with fibronectin in semen, and this complex exhibits superior viral enhancing activity over amyloids alone. The interface between fibronectin and semen amyloids can therefore serve as a novel microbicide target

- Completion of primary screen for semen amyloid deaggregators that decrease the ability of semen amyloids to enhance HIV infection

REPORTABLE OUTCOMES

- Manuscripts
    - “Semen amyloids that enhance HIV-1 infection are conserved in primates and play a physiological role in temporarily immobilizing spermatozoa.” Roan NR, Liu H, Usmani SM, Müller JA, Avila-Herrara A, Hamil K, Rosen J, Gawanbacht A, Zirafi O, Chu S, Neidleman J, Dong M, Smith JF, Pollard KS, O’Rand M, Lishko P, Kirchhoff F, Münch J, Witkowska HE, Greene WC. *Under review.*
    - “Fibronectin augments amyloid-mediated enhancement of HIV infection.” Roan NR, Chu S, Liu H, Witkowska HE, Greene WC. *Manuscript in preparation.*
    - “Seminal plasma induces global transcriptomic and immunosecretoy changes in endometrial epithelial cells and stromal fibroblasts associated with immune cell trafficking, angiogenesis, and proliferation.” Chen JC, Johnson BA, Erikson DW, Piltonen TT,

Barragan F, Chu S, Irwin JC, Greene WC, Giudice LC Roan NR. *Manuscript in preparation.*

- "Semen-mediated enhancement of HIV infection markedly impairs the antiviral efficacy of microbicdes." Kim KA, Roan NR, Zirafi O, Kluge SF, Jiang S, Greene WC, Kirchhoff F, Münch J. *Under revision (Sci Trans Med)*
- "Peptides released by physiological cleavage of semen coagulum proteins form amyloids that enhance HIV infection." Roan NR, Müller JA, Liu H, Chu S, Arnold F, Stürzel CM, Walther P, Dong M, Witkowska HE, Kirchhoff F, Münch J, Greene WC. *Cell Host Microbe*. 2011 Dec 15;10(6):541-50. PMID: 22177559
- [Role of semen-derived amyloid fibrils as facilitators of HIV infection]. Roan NR, Cavrois M, Greene WC. *Med Sci* (31). 2012 Apr;28(4):358-60. Epub 2012 Apr 25. French. PMID: 22549858

- Abstracts / Presentations
  - Roan N.R. "Semen amyloids, HIV transmission, and Fertility." Invited seminar at BIRCWH seminar series, University of California San Francisco, May 2013.
  - Roan N.R. Oral presentation at the 19th International AIDS Conference, Washington D.C., July 2012.
  - Roan N.R. "Identification and characterization of amyloid fibrils from human semen that enhance HIV infection". Invited seminar at Symposium of the International Graduate School in Molecular Medicine, Ulm University. March 2012.
  - Roan N.R. "Identification and characterization of amyloid fibrils from human semen that enhance HIV infection". Invited seminar at Jiaotong University School of Medicine, Shanghai, China. November 2011.
  - Roan N.R. "Identification and characterization of amyloid fibrils from human semen that enhance HIV infection". Invited seminar at University of California, Berkeley, Infectious Diseases and Immunity seminar series. October 2011.

CONCLUSIONS

The overall goal of this proposal is to better understand how semen and semen amyloids promote HIV transmission, and to identify ways to block this activity. During the first two years of the project, we have succeeded in identifying and characterizing novel viral enhancing factors from semen. We studied the ability of individual enhancing factors as well as semen in its entirety to enhance HIV infection directly, or indirectly by promoting inflammation including IL-6 production. We further studied these factors in semen samples from HIV-infected men, and provided evidence that semen amyloids can promote HIV transmission *in vivo.* The practical application of understanding the activity of these amyloids is to identify novel ways to prevent HIV transmission. To this

end, we have completed the first stage of a small molecule screen for inhibitors of the viral enhancing activity of semen, and have identified a list of candidates. Pending Year 3 funding, we will test the potency of the compounds to inhibit semen-mediated HIV transmission both *in vitro* and *in vivo*, using the models we established in the first two years of funding. Such knowledge can lead directly to the identification of useful usable drugs for HIV prevention efforts.

Appendix

None

References

1. Abdool Karim SS, Richardson BA, Ramjee G, Hoffman IF, Chirenje ZM, Taha T, Kapina M, Maslankowski L, Coletti A, Profy A, Moench TR, Piwowar-Manning E, Masse B, Hillier SL, Soto-Torres L. (2011) Safety and effectiveness of BufferGel and 0.5% PRO2000 gel for the prevention of HIV infection in women. *Aids* 25:957-966. 3083640
2. Munch J, Rucker E, Standker L, Adermann K, Goffinet C, Schindler M, Wildum S, Chinnadurai R, Rajan D, Specht A, Gimenez-Gallego G, Sanchez PC, Fowler DM, Koulov A, Kelly JW, Mothes W, Grivel JC, Margolis L, Keppler OT, Forssmann WG, Kirchhoff F. (2007) Semen-derived amyloid fibrils drastically enhance HIV infection. *Cell* 131:1059-1071.
3. Hauber I, Hohenberg H, Holstermann B, Hunstein W, Hauber J. (2009) The main green tea polyphenol epigallocatechin-3-gallate counteracts semen-mediated enhancement of HIV infection. *Proc Natl Acad Sci U S A* 106:9033-9038.
4. Roan NR, Munch J, Arhel N, Mothes W, Neidleman J, Kobayashi A, Smith-McCune K, Kirchhoff F, Greene WC. (2009) The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. *J Virol* 83:73-80.
5. Roan NR, Sowinski S, Munch J, Kirchhoff F, Greene WC. (2010) Aminoquinoline surfen inhibits the action of SEVI (semen-derived enhancer of viral infection). *J Biol Chem* 285:1861-1869.
6. Kim KA, Yolamanova M, Zirafi O, Roan NR, Staendker L, Forssmann WG, Burgener A, Dejucq-Rainsford N, Hahn BH, Shaw GM, Greene WC, Kirchhoff F, Munch J. (2010) Semen-mediated enhancement of HIV infection is donor-dependent and correlates with the levels of SEVI. *Retrovirology* 7:55.
7. Olsen JS, Brown C, Capule CC, Rubinshtein M, Doran TM, Srivastava RK, Feng C, Nilsson BL, Yang J, Dewhurst S. (2010) Amyloid-binding small molecules efficiently block SEVI (semen-derived enhancer of virus infection)- and semen-mediated enhancement of HIV-1 infection. *J Biol Chem* 285:35488-35496.
8. Roan NR, Muller JA, Liu H, Chu S, Arnold F, Sturzel CM, Walther P, Dong M, Witkowska HE, Kirchhoff F, Munch J, Greene WC. (2011) Peptides Released by Physiological Cleavage of Semen Coagulum Proteins Form Amyloids that Enhance HIV Infection. *Cell host & microbe* 10:541-550.

9. Arnold F, Schnell J, Zirafi O, Sturzel C, Meier C, Weil T, Standker L, Forssmann WG, Roan NR, Greene WC, Kirchhoff F, Munch J. (2011) Naturally Occurring Fragments from Two Distinct Regions of the Prostatic Acid Phosphatase Form Amyloidogenic Enhancers of HIV Infection. *Journal of virology*
10. Hartjen P, Frerk S, Hauber I, Matzat V, Thomssen A, Holstermann B, Hohenberg H, Schulze W, Schulze Zur Wiesch J, van Lunzen J. (2012) Assessment of the range of the HIV-1 infectivity enhancing effect of individual human semen specimen and the range of inhibition by EGCG. *AIDS research and therapy* 9:2.
11. Robert M, Gibbs BF, Jacobson E, Gagnon C. (1997) Characterization of prostate-specific antigen proteolytic activity on its major physiological substrate, the sperm motility inhibitor precursor/semenogelin I. *Biochemistry* 36:3811-3819.
12. Hu J, Gardner MB, Miller CJ. (2000) Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. *Journal of virology* 74:6087-6095. 112107
13. Udan ML, Ajit D, Crouse NR, Nichols MR. (2008) Toll-like receptors 2 and 4 mediate Abeta(1-42) activation of the innate immune response in a human monocytic cell line. *J Neurochem* 104:524-533.
14. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, Fitzgerald KA, Latz E, Moore KJ, Golenbock DT. (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9:857-865.
15. Tukel C, Wilson RP, Nishimori JH, Pezeshki M, Chromy BA, Baumler AJ. (2009) Responses to amyloids of microbial and host origin are mediated through toll-like receptor 2. *Cell Host Microbe* 6:45-53.
16. Chen J, Zhou Y, Mueller-Steiner S, Chen LF, Kwon H, Yi S, Mucke L, Gan L. (2005) SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. *J Biol Chem* 280:40364-40374.
17. Gutsche S, von Wolff M, Strowitzki T, Thaler CJ. (2003) Seminal plasma induces mRNA expression of IL-1beta, IL-6 and LIF in endometrial epithelial cells in vitro. *Molecular human reproduction* 9:785-791.
18. Robertson SA. (2005) Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res* 322:43-52.
19. Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA. (2007) Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. *Mol Hum Reprod* 13:491-501.
20. Perkins ND, Edwards NL, Duckett CS, Agranoff AB, Schmid RM, Nabel GJ. (1993) A cooperative interaction between NF-kappa B and Sp1 is required for HIV-1 enhancer activation. *Embo J* 12:3551-3558.
21. West MJ, Lowe AD, Karn J. (2001) Activation of human immunodeficiency virus transcription in T cells revisited: NF-kappaB p65 stimulates transcriptional elongation. *J Virol* 75:8524-8537.
22. Herbein G, Gras G, Khan KA, Abbas W. Macrophage signaling in HIV-1 infection. *Retrovirology* 7:34.

23. Rollenhagen C, Asin SN. Enhanced HIV-1 replication in ex vivo ectocervical tissues from post-menopausal women correlates with increased inflammatory responses. *Mucosal Immunol* 4:671-681.
24. Nazli A, Chan O, Dobson-Belaire WN, Ouellet M, Tremblay MJ, Gray-Owen SD, Arsenault AL, Kaushic C. (2010) Exposure to HIV-1 directly impairs mucosal epithelial barrier integrity allowing microbial translocation. *PLoS Pathog* 6:e1000852.
25. Suzuki T, Yoshinaga N, Tanabe S. (2011) Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *The Journal of biological chemistry* 286:31263-31271. 3173073
26. Adams RB, Planchon SM, Roche JK. (1993) IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. *Journal of immunology* 150:2356-2363.
27. Colgan SP, Resnick MB, Parkos CA, Delp-Archer C, McGuirk D, Bacarra AE, Weller PF, Madara JL. (1994) IL-4 directly modulates function of a model human intestinal epithelium. *Journal of immunology* 153:2122-2129.
28. van den Berg RH, Faber-Krol MC, Sim RB, Daha MR. (1998) The first subcomponent of complement, C1q, triggers the production of IL-8, IL-6, and monocyte chemoattractant peptide-1 by human umbilical vein endothelial cells. *Journal of immunology* 161:6924-6930.
29. Olsen A, Jonsson A, Normark S. (1989) Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. *Nature* 338:652-655.
30. Bozzini S, Falcone V, Conaldi PG, Visai L, Biancone L, Dolei A, Toniolo A, Speziale P. (1998) Heparin-binding domain of human fibronectin binds HIV-1 gp120/160 and reduces virus infectivity. *Journal of medical virology* 54:44-53.
31. Potash MJ, Chao W, Bentsman G, Paris N, Saini M, Nitkiewicz J, Belem P, Sharer L, Brooks AI, Volsky DJ. (2005) A mouse model for study of systemic HIV-1 infection, antiviral immune responses, and neuroinvasiveness. *Proc Natl Acad Sci U S A* 102:3760-3765. 553332

Supporting Data

Figure 1. Fresh semen samples enhance HIV infection but activity declines with increasing liquefaction time. Fresh ejaculate was liquefied for the indicated number of minutes before an aliquot was removed, pre-treated with HIV-1 for 5 min, and then diluted 15-fold and added to TZM-bl cells. Medium was replaced after 2 hr, and cells were assayed for Tat-inducible β-galactosidase activity 3 days later. RLU/s: relative light units / second.

Figure 2. A progressive decrease in viral enhancement activity coincides with the degradation of SEM fragments during prolonged liquefaction. *(A)* To assess the effect of prolonged liquefaction on semen's ability to enhance HIV infection, a stock solution of seminal fluid was incubated for the indicated periods of time, then mixed with 81A virions for 5 min, diluted 15-fold and added to TZM-bl cells. Medium was replaced after 2 hr, and cells were assayed for Tat-inducible β-galactosidase activity 3 days later. RLU/s: relative light units / second. Shown are average representative values (± std dev) of triplicate measurements from one of ten independent experiments. *(B)* Seminal fluid samples treated as described in (A) were analyzed by Western blot with the indicated primary antibodies.

Figure 3. The serine protease inhibitor AEBSF restores both HIV enhancing activity and SEM degradation in seminal fluid during prolonged semen liquefaction. *(A)* The seminal fluid stock solution was treated and assayed as described in Fig. 2, except that treatment was carried out in the absence or presence of the serine protease inhibitor AEBSF (5 mM). RLU/s: relative light units / second. Shown are average representative values (± std dev) of triplicate measurements from one of eight independent experiments. *(B)* Seminal fluid samples treated as described in (A) was analyzed by immunoblotting with the indicated primary antibodies. The steady levels of albumin and lactoferrin in all samples served as an internal control.

Figure 4. Fibronectin binds semen amyloids. (A) Label-free mass spectrometry quantitation of fibronectin bound to biotinylated SEM amyloids, beads only, or the monomeric SEM peptide. NSAF = normalized spectral abundance factor. (B) SEM amyloids, but not monomeric SEM peptide, bind purified FN. Beads conjugated to the indicated materials were incubated with FN, washed, and then assessed by dot blot (*top*) or Western blot (*bottom*) analysis.

Figure 5. FN augments the ability of semen amyloids to enhance HIV-1 infection. (A-F) CCR5-tropic 81A HIV-1 virions (100 ng/ml $p24^{Gag}$) were incubated for 5 min with the indicated concentrations of the corresponding factors, diluted 15-fold, and then added to TZM-bl cells. Infection was assessed three days later by luminescence. Background signal corresponding to uninfected cells was subtracted from shown values. (A) FN inhibits HIV-1 infection of TZM-bl cells. *$p<0.05$, ****$p<0.0001$ (Bonferonni multiple comparison

test as compared infection in the absence of FN). (B) FN increases the ability of SEM amyloids to enhance HIV-1 infection. (C) Same as in *(B)*, additionally showing the theoretical additive value of infection levels in the presence of FN or SEM amyloids alone (grey line). The actual infection observed in the presence of both FN and SEM amyloids (purple) is higher than the theoretical value, demonstrating a synergistic effect. (D) FN increases the ability of SEVI to enhance HIV-1 infection. (E) FN and albumin do not enhance HIV infection. (F) SEM and SEVI amyloids do not synergistically enhance HIV-1 infection. (G-H) HIV-1 infection of CEM.M7 cells. CCR5-tropic 81A HIV-1 virions (100 ng/ml $p24^{Gag}$) were incubated for 5 min with the indicated concentrations of the corresponding factors, diluted 15-fold, and then added to suspension CEM.M7 cells. Infection was assessed three days later by analysis on a LSRII flow cytometer . FN synergistically increases the ability of SEM amyloids (G) and SEVI (H) to enhance HIV-1 infection of CEM.M7 cells.

Figure 6. FN increases the ability of SEM amyloids to enhance both CCR5-tropic and CXCR4-tropic HIV-1 infection of CD4+ PBLs. CD14-CD4+ cells were purified from buffy coats, activated with PHA/IL-2. Two days later, nef-IRES-GFP HIV-1 virions (20 ng/ml $p24^{Gag}$) were pre-treated for 5 min with the indicated concentration of FN or SEM amyloids, diluted 15-fold and added to the cells. Infection was assessed three days later by by analysis on a LSRII flow cytometer.

Figure 7. SEM amyloids do not alter the conformation of the amyloids, or increase their ability to bind target cells. (A) SEM amyloids (1 mg/ml) were incubated in the absence or presence of FN (1 mg/ml) and examined by EM. Scale bars = 20 μm. (B) 100 μg/ml SEM amyloids was incubated with HIV-1 81A (100 ng/ml $p24^{Gag}$) for 3 hr at 37°C. Following centrifugation, the absolute amounts of $p24^{Gag}$ in the pellet and supernatant were determined by ELISA. (C) TZM-bl cells were incubated for 1 hr at 37°C with 50 μg/ml Proteostat-stained SEM amyloids, washed with media, and then imaged by a Zeiss LSM510 confocal microscope at a total magnification of 250x. DAPI counterstaining was used to detect individual cells.

Figure 1

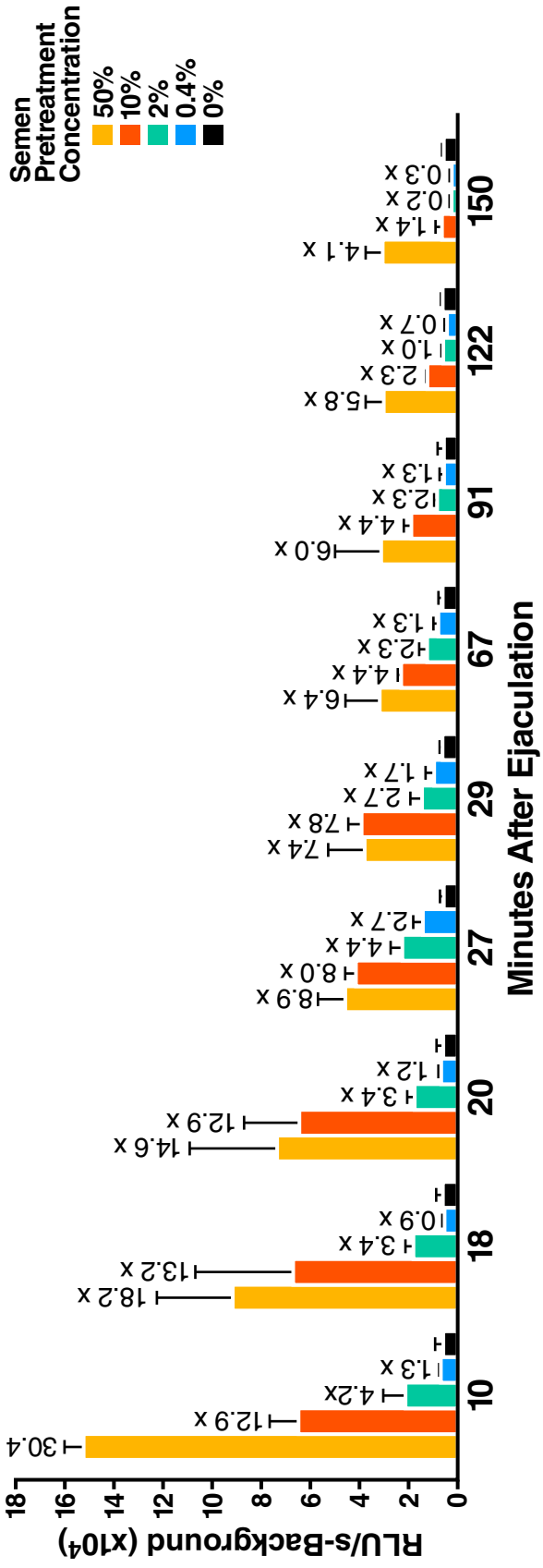
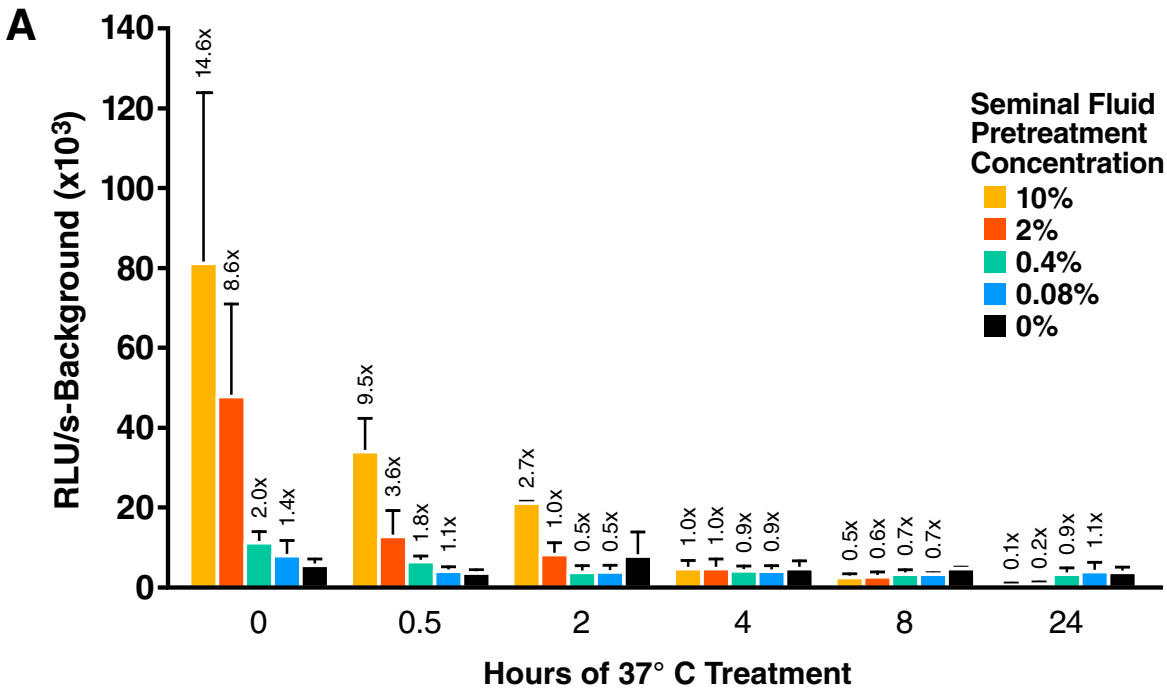

Semen Pretreatment Concentration
50%
10%
2%
0.4%
0%
RLU/s-Background (x10⁴)
0
2
4
6
8
10
12
14
16
18
Minutes After Ejaculation
10
18
20
27
29
67
91
122
150
30.4 x
12.9 x
4.2x
1.3 x
18.2 x
13.2 x
3.4 x
0.9 x
14.6 x
12.9 x
3.4 x
1.2 x
8.9 x
8.0 x
4.4 x
2.7 x
7.4 x
7.8 x
2.7 x
1.7 x
6.4 x
4.4 x
2.3 x
1.3 x
6.0 x
4.4 x
2.3 x
1.3 x
5.8 x
2.3 x
1.0 x
0.7 x
4.1 x
1.4 x
0.2 x
0.3 x

Figure 2


A
RLU/s-Background (x10³)
140
120
100
80
60
40
20
0
14.6x
8.6x
2.0x
1.4x
9.5x
3.6x
1.8x
1.1x
2.7x
1.0x
0.5x
0.5x
1.0x
1.0x
0.9x
0.9x
0.5x
0.6x
0.7x
0.7x
0.1x
0.2x
0.9x
1.1x
0
0.5
2
4
8
24
Hours of 37° C Treatment
Seminal Fluid Pretreatment Concentration
10%
2%
0.4%
0.08%
0%

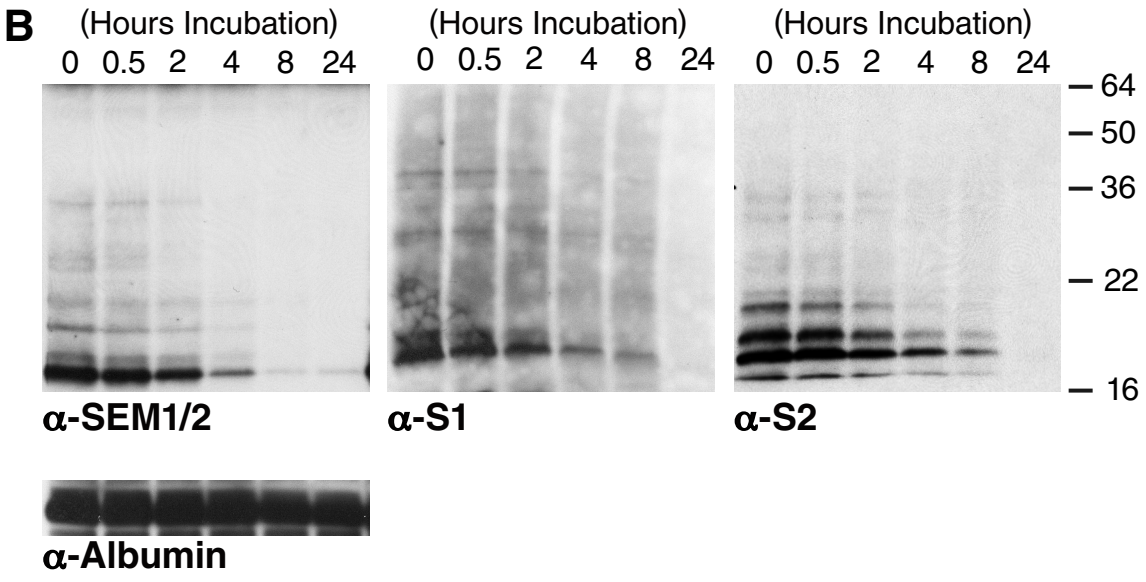
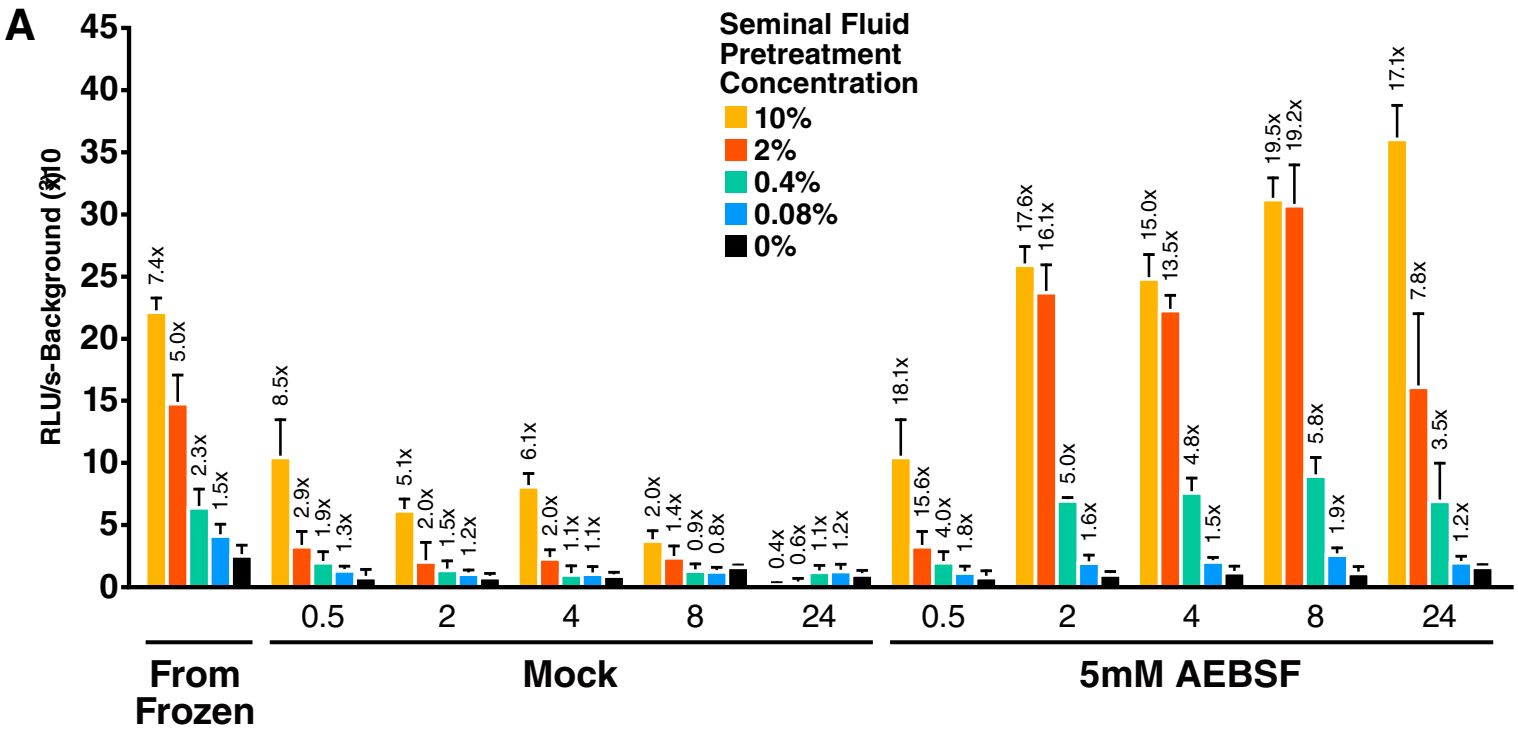

B
(Hours Incubation)
0 0.5 2 4 8 24
(Hours Incubation)
0 0.5 2 4 8 24
(Hours Incubation)
0 0.5 2 4 8 24
64
50
36
22
16
α-SEM1/2
α-S1
α-S2
α-Albumin

Figure 3


A
RLU/s-Background (X10
Seminal Fluid Pretreatment Concentration
10%
2%
0.4%
0.08%
0%
7.4x
5.0x
2.3x
1.5x
8.5x
2.9x
1.9x
1.3x
5.1x
2.0x
1.5x
1.2x
6.1x
2.0x
1.1x
1.1x
2.0x
1.4x
0.9x
0.8x
0.4x
0.6x
1.1x
1.2x
18.1x
15.6x
4.0x
1.8x
17.6x
16.1x
5.0x
1.6x
15.0x
13.5x
4.8x
1.5x
19.5x
19.2x
5.8x
1.9x
17.1x
7.8x
3.5x
1.2x
0.5
2
4
8
24
From Frozen
Mock
5mM AEBSF

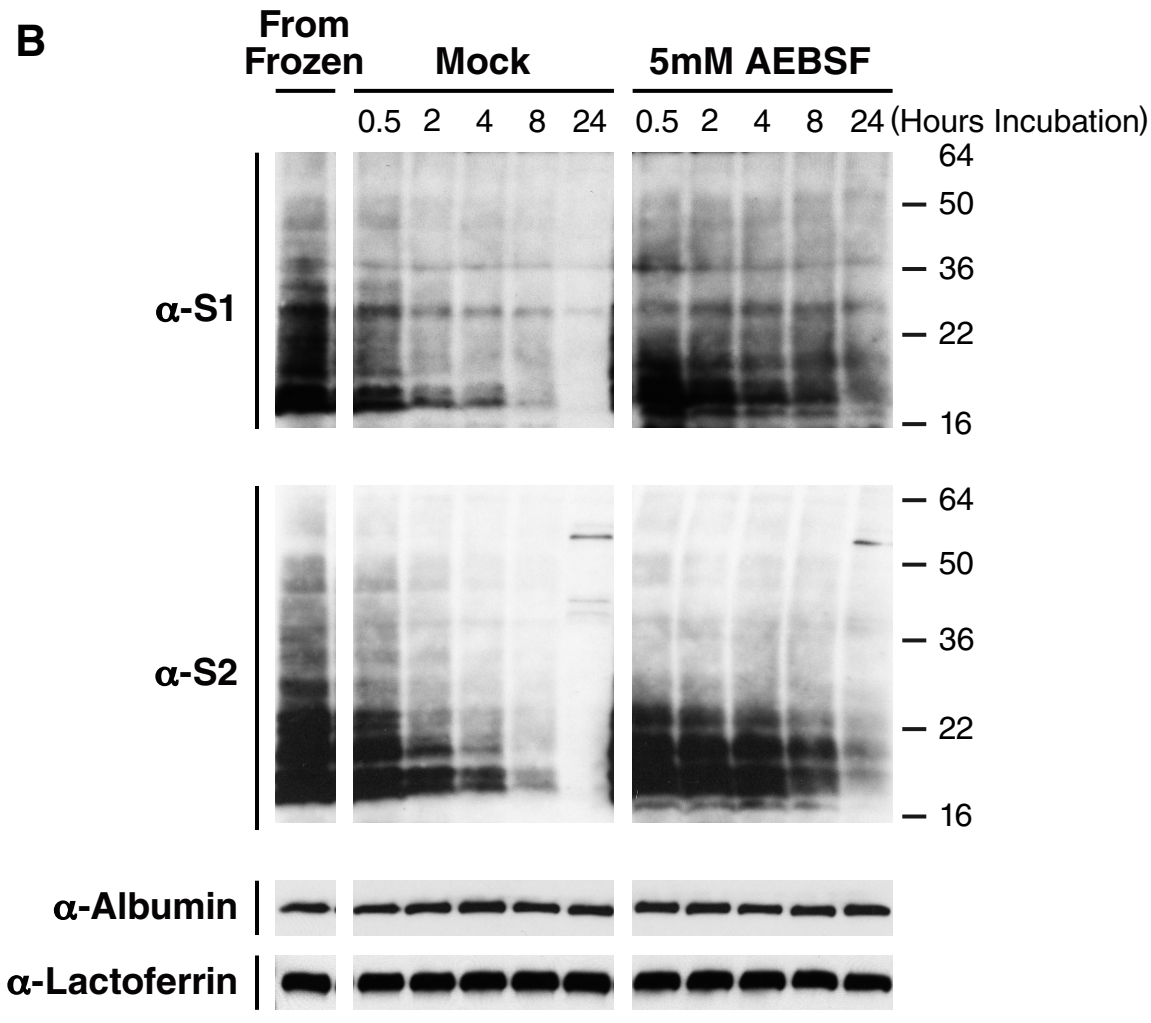

B
From Frozen
Mock
5mM AEBSF
0.5 2 4 8 24 0.5 2 4 8 24 (Hours Incubation)
α-S1
α-S2
α-Albumin
α-Lactoferrin
64
50
36
22
16

Figure 4

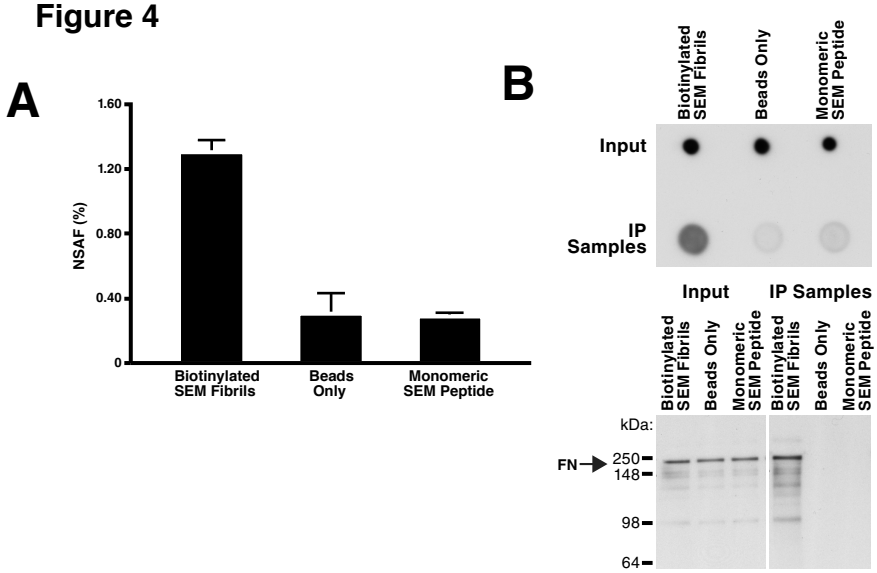

A
1.60
1.20
0.80
0.40
0
NSAF (%)
Biotinylated SEM Fibrils
Beads Only
Monomeric SEM Peptide
B
Biotinylated SEM Fibrils
Beads Only
Monomeric SEM Peptide
Input
IP Samples
Input
IP Samples
Biotinylated SEM Fibrils
Beads Only
Monomeric SEM Peptide
Biotinylated SEM Fibrils
Beads Only
Monomeric SEM Peptide
kDa:
FN
250
148
98
64

Figure 5

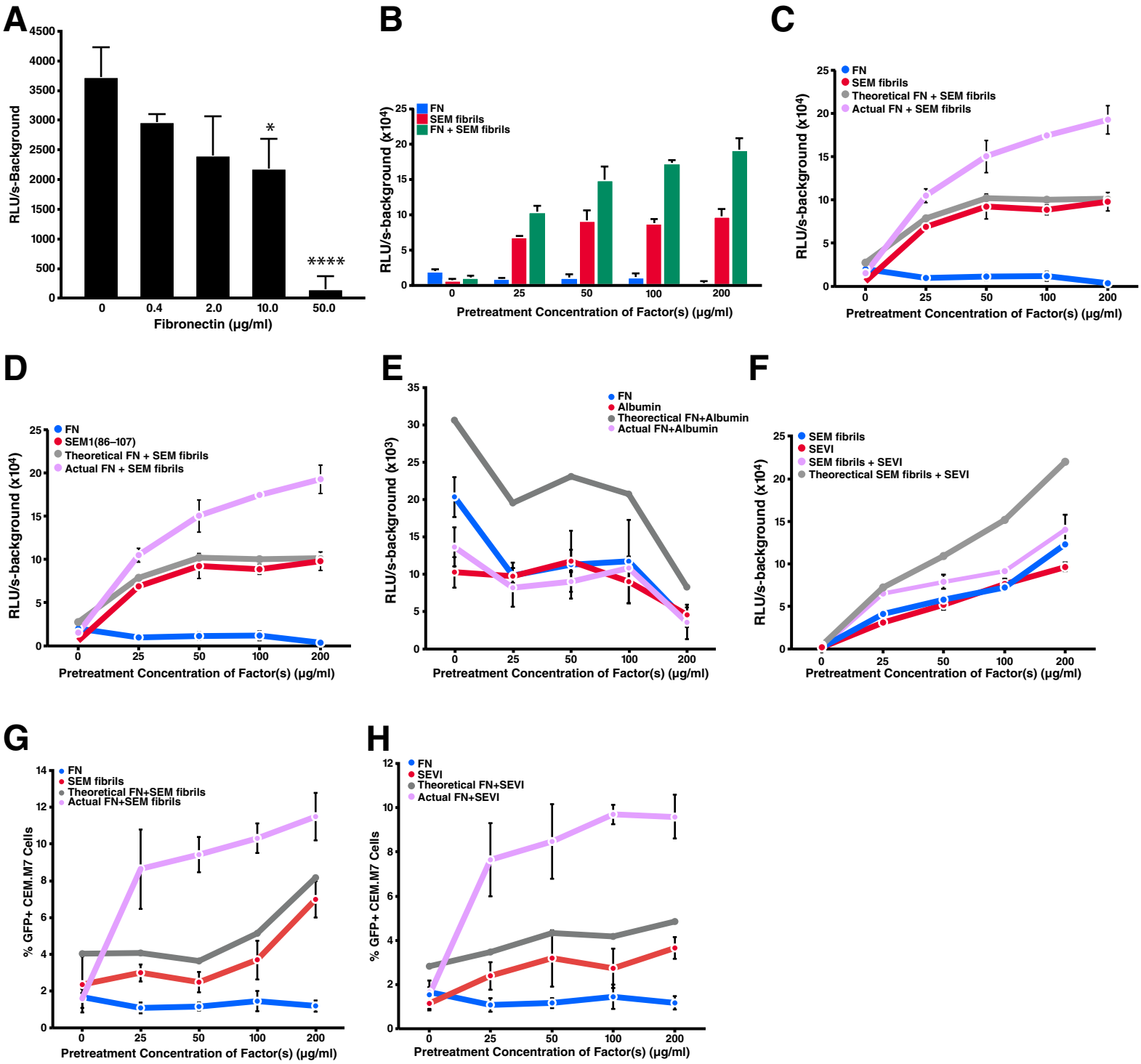

A
RLU/s-Background
Fibronectin (μg/ml)
0
0.4
2.0
10.0
50.0
*
****
B
RLU/s-background (x10⁴)
FN
SEM fibrils
FN + SEM fibrils
Pretreatment Concentration of Factor(s) (μg/ml)
C
RLU/s-background (x10⁴)
FN
SEM fibrils
Theoretical FN + SEM fibrils
Actual FN + SEM fibrils
Pretreatment Concentration of Factor(s) (μg/ml)
D
RLU/s-background (x10⁴)
FN
SEM1(86–107)
Theoretical FN + SEM fibrils
Actual FN + SEM fibrils
Pretreatment Concentration of Factor(s) (μg/ml)
E
RLU/s-background (x10³)
FN
Albumin
Theoretical FN+Albumin
Actual FN+Albumin
Pretreatment Concentration of Factor(s) (μg/ml)
F
RLU/s-background (x10⁴)
SEM fibrils
SEVI
SEM fibrils + SEVI
Theoretical SEM fibrils + SEVI
Pretreatment Concentration of Factor(s) (μg/ml)
G
% GFP+ CEM.M7 Cells
FN
SEM fibrils
Theoretical FN+SEM fibrils
Actual FN+SEM fibrils
Pretreatment Concentration of Factor(s) (μg/ml)
H
% GFP+ CEM.M7 Cells
FN
SEVI
Theoretical FN+SEVI
Actual FN+SEVI
Pretreatment Concentration of Factor(s) (μg/ml)

Figure 6

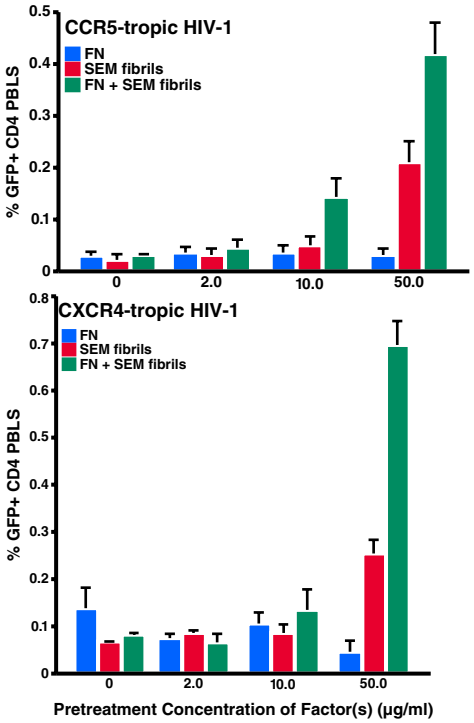
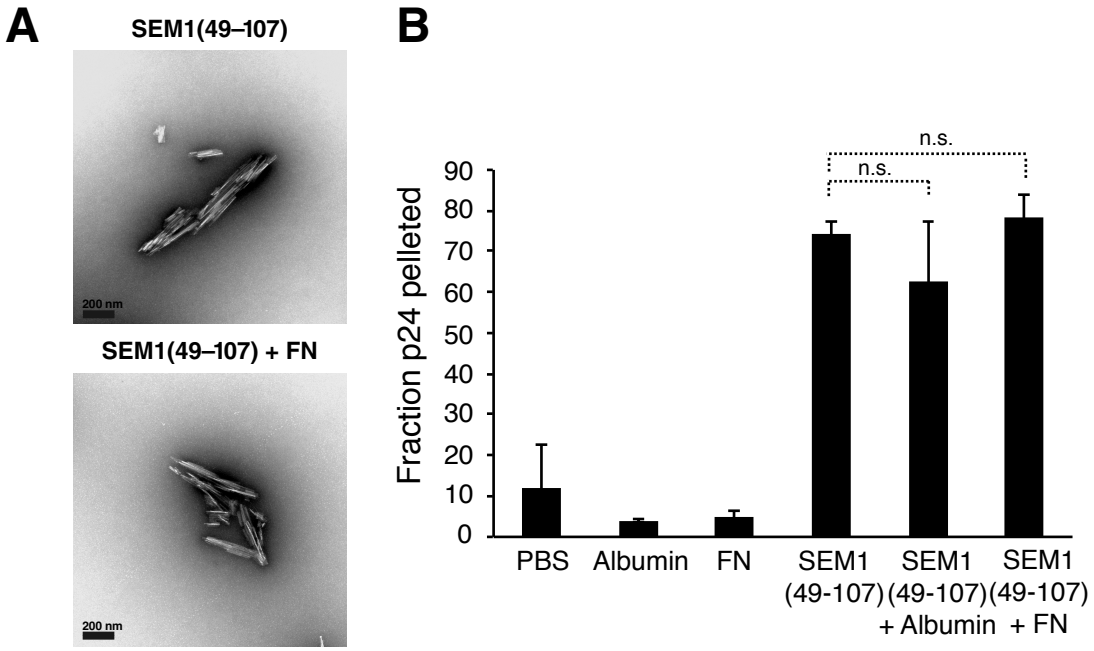
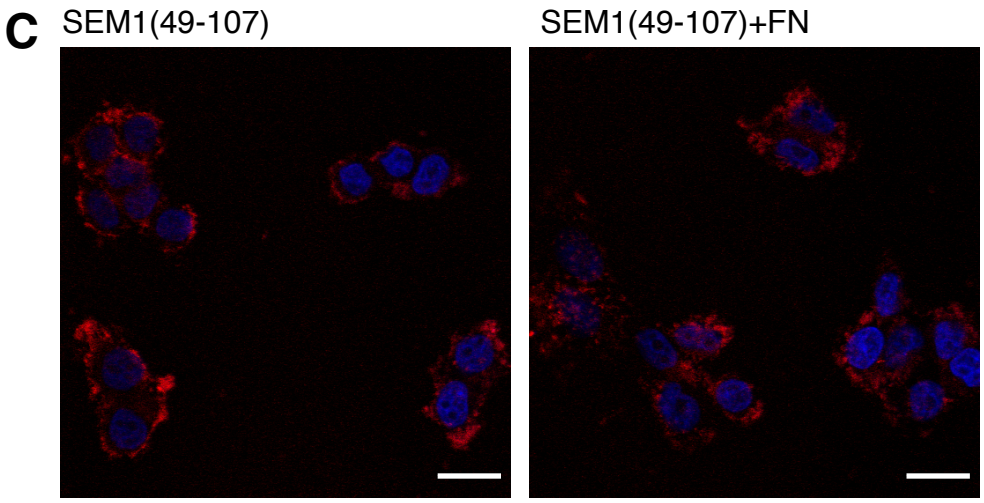

CCR5-tropic HIV-1
FN
SEM fibrils
FN + SEM fibrils
% GFP+ CD4 PBLS
0.5
0.4
0.3
0.2
0.1
0
0
2.0
10.0
50.0
CXCR4-tropic HIV-1
FN
SEM fibrils
FN + SEM fibrils
% GFP+ CD4 PBLS
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2.0
10.0
50.0
Pretreatment Concentration of Factor(s) (µg/ml)

Figure 7


A
SEM1(49–107)
200 nm
SEM1(49–107) + FN
200 nm
B
Fraction p24 pelleted
90
80
70
60
50
40
30
20
10
0
n.s.
n.s.
PBS
Albumin
FN
SEM1 (49-107)
SEM1 (49-107) + Albumin
SEM1 (49-107) + FN


C
SEM1(49-107)
SEM1(49-107)+FN